The Plant Cell, Vol. 21: 1647–1658, June 2009, www.plantcell.org

RESEARCH ARTICLES

The Maize Transcription Factor KNOTTED1 Directly Regulates the Gibberellin Catabolism Gene *ga2ox1* [W] [OA]

Nathalie Bolduc and Sarah Hake[1]

Plant Gene Expression Center, U.S. Department of Agriculture–Agricultural Research Service, University of California, Albany, California 94710

KNOTTED1 (KN1)-like homeobox (KNOX) transcription factors are involved in the establishment and maintenance of plant meristems; however, few direct targets of KNOX proteins have been recognized. Using a combination of double mutant analysis and biochemistry, we found that in maize (*Zea mays*), KN1 negatively modulates the accumulation of gibberellin (GA) through the control of *ga2ox1*, which codes for an enzyme that inactivates GA. The *ga2ox1* mRNA level is elevated in immature leaves of dominant KNOX mutants and downregulated in reproductive meristems of the null allele *kn1-e1*. KN1 binds in vivo to an intron of *ga2ox1* through a *cis*-regulatory element containing two TGAC motifs. VP16-KN1 activates transcription in planta from a chimeric promoter containing this binding site. The domains of expression of *kn1* and *ga2ox1* mRNAs overlap at the base of the shoot apical meristem and the base of newly initiated leaves, suggesting that KN1-mediated activation of *ga2ox1* maintains a boundary between meristem cell identity and rapidly elongating cells of the shoot. The KN1 binding site is conserved in *ga2ox1* genes of different grasses, suggesting that the local regulation of bioactive GA levels through KNOX proteins is a common theme in grasses.

INTRODUCTION

Organogenesis throughout the plant life cycle relies on the proper activity of a pool of stem cells located in meristems. Meristematic cell identity and maintenance require activity of class I KNOTTED1-like homeobox (KNOX) transcription factors, which include SHOOT MERISTEMLESS (STM) in *Arabidopsis thaliana* and KNOTTED1 (KN1) in maize (*Zea mays*). Severe *stm* mutants fail to progress in shoot development beyond the cotyledon stage (Long et al., 1996). In maize, loss-of-function *kn1* mutants, such as *kn1-e1*, are also impaired in shoot development in some inbred backgrounds (Vollbrecht et al., 2000). In permissive backgrounds, vegetative development remains mostly unaffected, but plants show severe developmental reproductive defects, including the occasional failure to develop a female inflorescence and reduced branching of both male and female inflorescences (Kerstetter et al., 1997; Vollbrecht et al., 2000). Gain-of-function mutations, such as *Kn1-N*, lead to misexpression of *kn1* in developing leaves, which promotes extra cell proliferation and outgrowth of cells, resulting in the formation of knots (Freeling and Hake, 1985; Smith et al., 1992).

knox genes belong to the TALE superclass of homeobox transcription factors found in plants and animals. The homeobox encodes a DNA binding domain, the homeodomain (HD), which folds in three α-helices and contacts the major groove of DNA through the third helix (Gehring et al., 1994). Similar to animal HD transcription factors, KNOX proteins recognize degenerate sites in vitro, which have hampered identification of downstream targets. In vitro, KNOX proteins bind sequences containing a TGAC core (Krusell et al., 1997; Smith et al., 2002; Tioni et al., 2005; Viola and Gonzalez, 2006).

Gibberellin (GA) promotes cell elongation and morphogenesis (Thomas et al., 2005), and according to the current model, KNOX proteins are needed to maintain low GA levels in the shoot apical meristem (SAM) (Hay et al., 2002; Jasinski et al., 2005). Indeed, KN1 homologs in tobacco (*Nicotiana tabacum*) and potato (*Solanum tuberosum*) directly bind to and repress a gene encoding a GA 20-oxidase (Sakamoto et al., 2001a; Chen et al., 2004), a rate-limiting enzyme in GA biosynthesis (Thomas et al., 2005). This repression results in *ga20ox* accumulation in leaves, where KNOX proteins are absent, but not in the SAM where they are abundant (Sakamoto et al., 2001a; Hay et al., 2002). In *Arabidopsis*, the *GA20ox1* mRNA level is reduced in leaves overexpressing the KNOX proteins STM or BREVIPEDICELLUS (Hay et al., 2002), but whether this regulation is direct awaits confirmation. Another hormonal pathway targeted by KNOX proteins is the cytokinin biosynthesis pathway (Ori et al., 1999). Cytokinins are plant growth regulators critical for meristem maintenance and they act antagonistically to GA (Greenboim-Wainberg et al., 2005). In *Arabidopsis*, the cytokinin synthesis gene *IPT7* is rapidly upregulated following overexpression of STM (Yanai et al., 2005). KNOX proteins may also antagonize cell differentiation by controlling the expression of cell wall biosynthesis genes (Mele et al., 2003; Groover et al., 2006). In these two cases, however, it

[1] Address correspondence to maizesh@nature.berkeley.edu.
The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Sarah Hake (maizesh@nature.berkeley.edu).
[W] Online version contains Web-only data.
[OA] Open access articles can be viewed online without a subscription.
www.plantcell.org/cgi/doi/10.1105/tpc.109.068221

remains unclear whether or not the activation is directly due to the binding of a KNOX protein.

Several lines of evidence indicate that KN1 regulates the GA pathway in maize. Dwarfism is generally associated with a defect in GA synthesis, perception, or metabolism (Thomas et al., 2005), and dominant *Kn1* mutants, such as *Kn1-N*, are shorter than their normal siblings. When *Kn1-N* mutants were supplemented with GA, the severity of the knotted phenotype (number of knots on leaves) was reduced, while it was aggravated when treating plants with an inhibitor of GA synthesis (Kessler et al., 2006). Accumulation of *ga2ox1* mRNA, which encodes an enzyme involved in inactivation of bioactive GA, is elevated in leaves of the dominant mutant *Kn1-N* (Kessler et al., 2006). *GA2ox2* mRNA level is similarly increased in *Arabidopsis* leaves overexpressing STM, but because *GA2ox2* mRNA is also upregulated in response to higher cytokinin levels, it remained unclear if the modulation was the indirect consequence of STM-induced cytokinin production (Jasinski et al., 2005). In this study, we use maize as a model organism to investigate the interplay between KNOX and GA in grasses. Our findings suggest that multiple aspects of the phenotypes conditioned by mutations at the *kn1* locus are modified when GA biosynthesis is inhibited. KN1 directly binds to a *cis*-regulatory element present in an intron of *ga2ox1* and thus favors the inactivation of bioactive GA. The presence of this *cis*-regulatory element in *ga2ox1* orthologs from other grasses suggests that this mechanism of local regulation of the GA pathway by KNOX proteins was positively selected during evolution.

RESULTS

Modification of *kn1* Gain- and Loss-of-Function Phenotypes by a Low GA Level

Given that KNOX proteins repress the accumulation of bioactive GA (Hay et al., 2002; Jasinski et al., 2005), we examined the effects of reduced GA synthesis on the phenotypes of mutations at the *kn1* locus in maize. We crossed the dominant mutant *Kn1-N* to the recessive mutant *dwarf3* (*d3*) and analyzed the F2 population. *d3* codes for an enzyme involved in one of the early steps of the GA biosynthesis pathway (Winkler and Helentjaris, 1995). The level of bioactive GA in *d3* plants is strongly reduced but not null (Phinney and Spray, 1982; Fujioka et al., 1988; Winkler and Helentjaris, 1995) so that further decrease is possible. The combination of the *d3* and *Kn1-N* mutations resulted in plants that were shorter than siblings carrying only one of the two mutations (Figure 1A, Table 1). Double mutant plants looked severely knotted (Figures 1A and 1B), with knots distributed in a smaller area, such that knot density was higher (Figure 1C). Similar observations were made when crossing *Kn1-N* to the dominant *Dwarf9* mutant (see Supplemental Figure 1 online). This finding is consistent with the increased knot formation observed after treatment of seedlings with a GA synthesis inhibitor (Kessler et al., 2006). In addition to the effect on plant height, the low GA level in *d3* plants generates wider and shorter leaves (Table 1). Leaves from *Kn1-N*/+ plants were as wide as those from *d3* siblings, and they became significantly wider in *d3; Kn1-N*/*Kn1-N* double mutants. Similarly, *d3 Kn1-N*/*Kn1-N* plants had shorter leaves than plants carrying only one of the two mutations (Table 1). Thus, multiple aspects of the phenotype observed in the gain-of-function *Kn1-N* mutant are enhanced when GA biosynthesis is inhibited, suggesting that repression of bioactive GA accumulation is instrumental to KN1 function.

If KN1 has a role in reducing bioactive GA during reproductive development, a reduction of the GA level should overcome some of the defects observed for loss-of-function mutations at the *kn1* locus. The phenotypic rescue would be substantial if the GA pathway is the main target of KN1 or subtle if the GA pathway is only one of numerous targets. To address this question, we crossed the null allele *kn1-e1* (Vollbrecht et al., 2000) to *d3* and analyzed the F2 population. One aspect of the *kn1-e1* phenotype is a reduction in the number of spikelets (flower-bearing branches) initiated by the male inflorescence (tassel) meristem (Kerstetter et al., 1997). Spikelet density in the tassel of *d3 kn1-e1* double

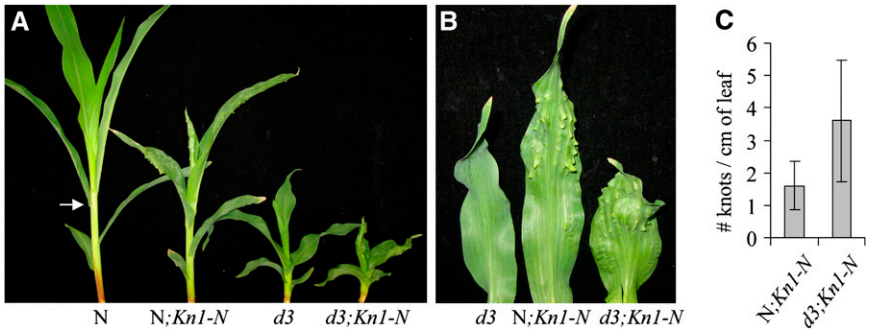

Figure 1. *Kn1-N* Phenotype Is Enhanced by a Low GA Level.

(A) to **(C)** *Kn1-N* phenotype at the seedling stage in an F2 family segregating *Kn1-N* and *d3*. *Kn1-N* plants are homozygous.

(A) General view of single and double mutants. Arrow points to the sheath/blade junction (auricle) of the third leaf, which was used as a reference point for height measurements in Table 1.

(B) Close-up view of leaf 3.

(C) Knot density in leaf 1 is higher when the *d3* and *Kn1-N* mutations are combined (P value = 0.027). Error bars represent the SD of 33 *N;Kn1-N* single mutants and nine *d3;Kn1-N* double mutants. N, d3/+ or +/+; d3, d3/d3.

Table 1. Comparison of the *Kn1-N* Phenotype in Normal and *d3* Backgrounds

Genotype	Width[a]	Length[a]	Height[b]
N; +/+	1.7 (0.2; 20)[c]	23.5 (2.6; 20)[c]	11.4 (0.8; 28)[c]
N; *Kn1-N*/+	2.1 (0.1; 26)[d]	23.0 (2.2; 26)[c]	10.9 (1.0; 41)[d]
N; *Kn1-N*/*Kn1-N*	2.8 (0.2; 18)[e]	17.2 (2.6; 18)[d]	10.1 (1.3; 33)[e]
d3; +/+	2.1 (0.2; 12)[d]	11.0 (1.2; 12)[e]	3.8 (0.4; 12)[f]
d3; *Kn1-N*/+	2.6 (0.2; 23)[f]	9.9 (1.1; 23)[f]	3.0 (0.4; 16)[g]
d3; *Kn1-N*/*Kn1-N*	3.4 (0.4; 14)[g]	6.6 (0.9; 14)[g]	2.6 (0.4; 7)[g]

All data are in centimeters. Number between parentheses are the SD followed by the number of individuals analyzed. Statistically significant differences between genotypes within each category are represented by different letters (c to g; P value < 0.01). N, *d3*/+ or +/+; *d3*, *d3*/*d3*.
[a]The distal part (blade) of the third leaf was measured at its widest and longest point.
[b]Distance from the soil to the sheath/blade junction of the third leaf.

mutants was higher than in *kn1-e1* plants but not as high as in normal siblings or the single *d3* mutants (Table 2; see Supplemental Figures 2A and 2B online). Thus, lowering the GA level in the tassel partially suppressed the *kn1-e1* phenotype, suggesting that KN1 acts upstream of multiple pathways, including GA.

In maize, the vegetative-to-reproductive transition is positively correlated with GA accumulation and signaling. Mutants impaired in GA biosynthesis flower late because they initiate more leaves before transitioning to the reproductive phase (Evans and Poethig, 1995). *kn1-e1* plants initiated fewer leaves than their normal siblings (Table 2), and the difference was statistically significant after introgression in the B73 inbred background (see Supplemental Table 1 online). The number of leaves produced in the *d3 kn1-e1* double mutants was also significantly less than *d3* alone (Table 2). These data suggest that accumulation of bioactive GA in the SAM in amounts favorable for the vegetative-to-reproductive transition occurs faster when KN1 is absent. In other words, KN1 delays flowering time, potentially by repressing the accumulation of bioactive GA in the SAM.

ga2ox1 mRNA Levels Correlate with the Presence or Absence of KNOX Proteins

The genetic evidence described above indicates that KN1 is involved in GA metabolism, while at the molecular level, a previous study showed that the *ga2ox1* mRNA level is increased in leaves of *Kn1-N* (Kessler et al., 2006). Since this gene encodes a GA catabolism enzyme, we hypothesized that it could represent one of the mechanistic links between KN1 and GA homeostasis. We used quantitative real-time RT-PCR to quantify *ga2ox1* mRNA in leaves of *Kn1-N* and two other dominant *knox* mutants, *Gn1-R* and *Lg3-O* (Foster et al., 1999; Muehlbauer et al., 1999). Similar to *Kn1* dominant mutants, misexpression of *gn1* and *lg3* in leaves leads to shorter plant stature, wider and shorter leaves, and extra cell proliferation and displacement of proximal cell fate into distal regions of the leaf. Confirming the findings of Kessler et al. (2006), *ga2ox1* was strongly upregulated in the leaves of *Kn1-N* (Figure 2). The *ga2ox1* mRNA level was also elevated in *Gn1-R* and *Lg3-O* leaves (Figure 2), suggesting that other KNOX proteins negatively regulate bioactive GA levels.

We also quantified *ga2ox1* mRNA in meristematic tissues where *kn1* normally accumulates. The highest accumulation was found in shoot apices (SAM-enriched dissected tissues), followed by ear and tassel primordia (Figure 2). The level of *ga2ox1* mRNA was significantly reduced in *kn1-e1* mutant tassels (P value = 0.014) and ears (P value = 0.016) but remained unchanged in *kn1-e1* shoot apices. Thus, in the B73 inbred background, the *ga2ox1* mRNA level is responsive to KN1 in reproductive meristems but not in vegetative meristems. Interestingly, *kn1-e1* plants show no major defect during their vegetative growth when in the B73 inbred, but their reproductive development is impaired (Kerstetter et al., 1997; Vollbrecht et al., 2000). The modulation of *ga2ox1* only in tissues most affected by the absence of KN1 (ears and tassels but not shoot apices) suggests that the modulation of *ga2ox1* mRNA level by KN1 has functional significance.

ga2ox1 and *kn1* mRNA Overlap in the SAM

If *ga2ox1* is a direct target of KN1, we expect to see overlap in their expression domains. We performed in situ hybridization on shoot apices to verify that *kn1* and *ga2ox1* mRNA colocalize. *ga2ox1* mRNA was detected at the base of the leaves as well as the base of the vegetative meristem and unexpanded stem (Figures 3B to 3D), with clear overlap with *kn1* mRNA (Figure 3A). This expression pattern is similar to those reported for *Arabidopsis GA2ox2* and *GA2ox4*, as well as rice (*Oryza sativa*) GA2ox1 (Sakamoto et al., 2001b; Jasinski et al., 2005). The same expression pattern was observed in *kn1-e1* shoot apices (see Supplemental Figure 3 online), suggesting that other KNOX proteins redundantly modulate *ga2ox1* and could compensate for the absence of KN1. Indeed, *gn1*, *lg3*, and other maize class I *knox* genes accumulate abundantly in shoot apices (Jackson et al., 1994; Schneeberger et al., 1995; Foster et al., 1999). Control sections hybridized with a sense-strand RNA probe showed no staining above background (Figure 3E).

KN1 Binds in Vivo to an Intron of the *ga2ox1* Gene

If KN1 directly regulates the expression of *ga2ox1*, KN1 should bind to a regulatory element present in the *ga2ox1* gene. We tested this hypothesis using chromatin immunoprecipitation (ChIP) with anti-KN1 antibodies that do not cross-react with

Table 2. Comparison of the *kn1-e1* Phenotype in Normal and *d3* Backgrounds

Genotype	No. of Leaves[a]	No. of Spikelets[b]	*n*
N; *kn1-e1*/+ or +/+	18.1 (0.9)[c]	63.5 (9.5)[c]	25
N; *kn1-e1*/ *kn1-e1*	17.7 (1.1)[c]	26.7 (7.8)[d]	30
d3; *kn1-e1*/+ or +/+	21.2 (1.6)[d]	71.8 (16.2)[e]	28
d3; *kn1-e1*/ *kn1-e1*	20.1 (1.5)[e]	33.4 (8.8)[f]	25

Values between parentheses are the SD. Statistically significant differences between genotypes within each category are represented by different letters (c to f; P value < 0.05). N, *d3*/+ or +/+ ; *d3*, *d3*/*d3*.
[a]Total number of leaves produced before initiation of the tassel.
[b]Spikelets were counted from the central 5 cm in the middle of the main rachis of the tassel.

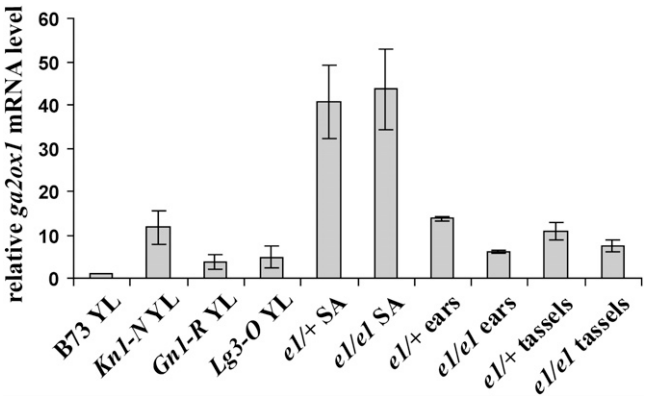

Figure 2. Expression Analysis of *ga2ox1* mRNA in Different Tissues and Genotypes.

The mRNA accumulation of *ga2ox1* was evaluated by real-time Q-RT-PCR using *gapdh* as the reference gene. Results are plotted as the ratio to the *ga2ox1* mRNA level detected in B73 YL. Error bars represent the SD between two to four biological replicates. YL, young leaves; SA, shoot apices (dissected tissue that is SAM enriched but also includes leaf primordia and developing stem); *e1*, *kn1-e1*.

other KNOX proteins (see Supplemental Figure 4 online). For convenience, we initially used ears of the double mutant *branched silkless1 Tunicate* (*bd1 Tu*), which makes excessive amounts of meristematic tissue (Chuck et al., 2002). ChIP samples were tested using quantitative PCR (Q-PCR) with primer pairs spanning different parts of the gene, referred to as *a* to *g* (Figure 4A). The strongest enrichment was obtained using primer pair *e* (Figure 4B). The same enrichment was consistently observed from different chromatin samples immunoprecipitated using KN1 antibodies but not from mock-treated chromatin samples. Regions of the gene ~4 kb upstream (*a*) or downstream (*g*) of region *e* showed very low enrichment, similar to that observed for the promoter of an α-tubulin (*tub*) gene not expected to be a KN1 target. Surprisingly, the region that contains the consensus in vitro KN1 binding site TGACAG(G/C)T (Smith et al., 2002), amplified with primer pair *c*, was not strongly enriched. To determine if the binding is specific to KN1, we repeated the ChIP assay on chromatin samples isolated from B73 plants and *kn1* mutants introgressed into B73. Q-PCR analysis performed using primer pair *e* showed an enrichment in B73 ears similar to that of *bd1 Tu* ears (Figure 4C). We detected a modest enrichment in normal tassels, normal shoot apices and *Kn1-N* leaves, but no enrichment in normal leaves or meristematic tissues dissected from plants carrying the *kn1-e1* null allele (Figure 4C). These results confirm the specificity of the ChIP assay and indicate that KN1 binds in vivo to regulatory elements near the 3′ end of the first intron of *ga2ox1* in all tissues where KN1 accumulates.

KN1, GN1, and LG3 Bind to *ga2ox1* through a *cis*-Regulatory Element Comprising Two TGAC Motifs

To define the *cis*-regulatory element that mediates KN1 binding to *ga2ox1*, we searched for putative KN1 binding sites in the DNA sequence delimited by primer pair *e* and identified three sites that contained at least the TGAC core of the consensus in vitro binding site (BS) (Figure 5A). We performed an electrophoretic mobility shift assay (EMSA) to test the ability of KN1 to bind in vitro to these putative *cis*-regulatory elements. The part of region *c* that contained a perfect in vitro consensus binding site was also included for comparison (Figure 5A, BS1). We

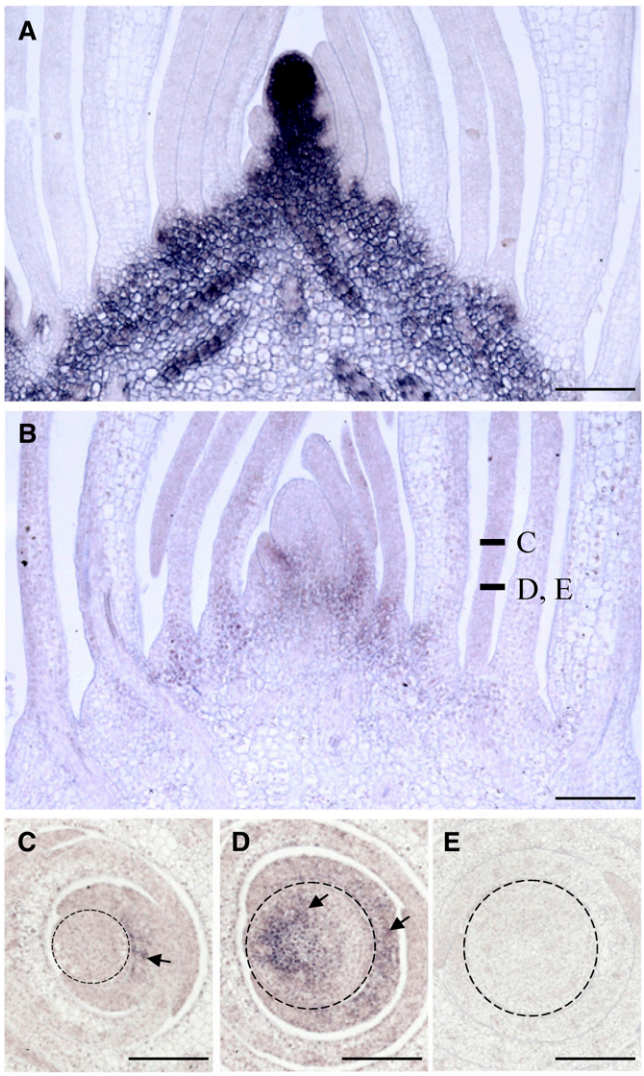

Figure 3. *ga2ox1* and *kn1* mRNA Overlap in the SAM.

(A) and **(B)** Longitudinal sections through B73 SAM.
(A) In situ hybridization with antisense *kn1* probe.
(B) In situ hybridization with antisense *ga2ox1* probe. Blue staining is mainly visible on the outside portion of the developing stem and at the base of the leaves. Bars show the approximate location of the transverse sections shown in **(C)** to **(E)**.
(C) to **(E)** Transverse sections through B73 SAM. SAM is encircled with a discontinuous line.
(C) and **(D)** In situ hybridization on serial sections with antisense *ga2ox1* probe. Arrows point to staining in leaves and meristem.
(E) In situ hybridization with sense *ga2ox1* probe.

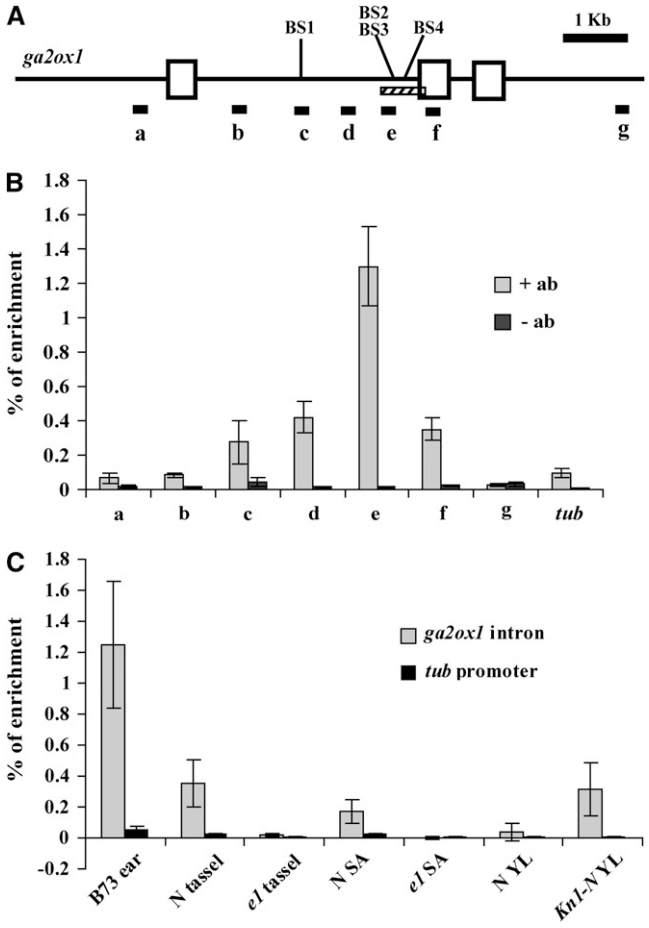

Figure 4. KN1 Binds in Vivo to the *ga2ox1* Gene.

(A) Cartoon representation of the *ga2ox1* gene structure. Exons are represented by white boxes, and letters show the approximate position of the fragments amplified in the ChIP DNA analysis. Dashed box shows the position of the fragment used for EMSA in Figure 5C.

(B) and **(C)** Q-PCR analysis of ChIP DNA enrichment performed with KN1-specific antibodies on ears from the *bd1;Tu* double mutant **(B)** or different maize tissues and genotypes in the B73 inbred background **(C)**. In **(C)**, only the intronic region *e* was analyzed. No enrichment was detected in any of the tissues using primers specific to the promoter of the nontarget gene *tubulin* (*tub*) or the *ga2ox1* promoter (region *a*, not shown). Enrichment is relative to the total DNA used in the input. Error bars represent the SD between two to three biological replicates. *e1, kn1-e1/kn1-e1;* N, normal siblings; YL, young leaves; SA, shoot apices (SAM enriched); ab, anti-KN1 antibodies.

expressed and purified from *Escherichia coli* fusion proteins between glutathione *S*-transferase (GST) and the homeodomain of KN1 (KN-HD). KN-HD was able to bind to all four DNA fragments to some degree (Figure 5B). However, the binding detected with BS4 was by far the strongest, suggesting that KN-HD has a substantially higher affinity for BS4 than for any of the other three fragments. These results suggested that the 30-bp region encompassed by BS4 contains the *cis*-regulatory elements bound in vivo by KN1.

To confirm that no other sequence conferred a significant KN-HD binding in region *e* of *ga2ox1*, we performed the EMSA with a 570-bp DNA fragment that covered region *e* plus sequences downstream (see hatched box, Figure 4A). The GST protein alone did not bind to this fragment, while KN-HD recognized the DNA probe as two distinct species: a major protein-DNA complex and a minor one of lower mobility (Figure 5C). The lower mobility protein-DNA complex is also visible with the BS4 probe (Figures 5B and 5E) and suggests that a small fraction of the probe is bound by two KN-HD molecules. To test the specificity of these interactions, we performed competition assays with the BS DNA fragments. Increasing concentrations of BS4 competed for KN-HD binding; however, a 500-fold molar excess of BS1, BS2, BS3, or mutated versions of BS4 (C1 and C2, see Figure 5A and text below) failed to compete for KN-HD binding (Figure 5C). These data indicate that BS4 contains most of the *cis*-regulatory elements that mediate KN1 binding to the 570-bp fragment.

To further verify the specificity of the EMSA assay, we used a mutant version of KN-HD in the recognition helix (KN* in Figures 5D and 5E) that abrogates DNA binding in KNOX proteins (see Methods). When using equal amounts of normal and mutant versions of the protein, the latter generated a substantially reduced binding to the BS4 probe (Figures 5D and 5E). These data indicate that the binding is specific to KN-HD and not due to contaminant proteins from *E. coli*.

Given that the *ga2ox1* mRNA level is elevated in other dominant *knox* mutants (Figure 2), we asked whether other maize KNOX proteins were able to bind to BS4. Using GST fusion proteins to the HD of GN1 (GN-HD) and LG3 (LG-HD), we detected strong binding of both to BS4 (Figure 5E). The specificity of these interactions was further tested using EMSA with mutated oligonucleotides as well as with competition assays. The G and A nucleotides of the TGAC motif are considered the most critical for KNOX-DNA interaction (Viola and Gonzalez, 2006). BS4 contains two TGAC core (C) motifs; thus, we used a BS4 fragment with mutations in either the first or the second motif, or both (referred to as BS4-C1, -C2, or -C1/2; Figure 5A) to address their involvement in HD recognition. KN-HD binding to BS4-C1 and BS4-C2 was very weak, and binding to BS4-C1/2 was undetectable (Figure 5E). In a competition assay, BS4 was able to compete for KN-HD binding, whereas BS4-C1 competed very weakly, and BS4-C2 or BS4-C1/2 was unable to compete (Figure 5F). We obtained very similar data with GN-HD and LG-HD (Figure 5G). These data indicate that the HD from KN1, GN1, and LG3 binds to the *ga2ox1 cis*-regulatory element with similar affinity and specificity and that both TGAC motifs are important for KNOX-HD binding.

In Planta Binding to the *cis*-Regulatory Element

To confirm that the KN1/KNOX binding site in the *ga2ox1* intron operates as a *cis*-regulatory element in planta, we performed an *Agrobacterium tumefaciens* transient assay in *Nicotiana benthamiana* leaves. Up to four copies of BS4 were introduced upstream of a minimal 35S promoter driving a firefly luciferase (FiLuc) reporter gene (Figure 6A). Full-length KN1 or a version lacking the HD (KN1ΔHD) with and without the activation domain

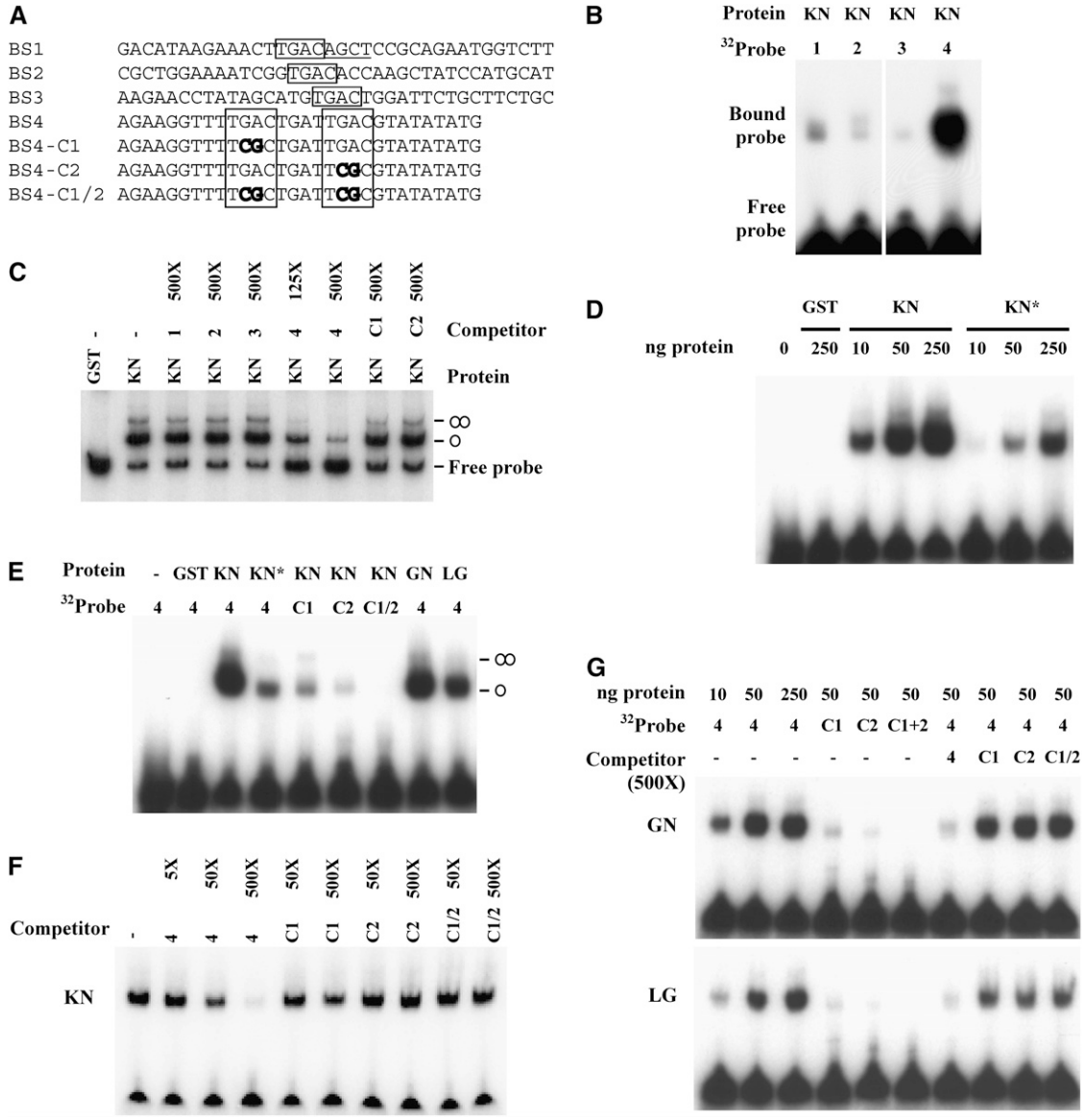

Figure 5. KN1 and Other KNOX Proteins Bind in Vitro to Specific Sequence Motifs in the *ga2ox1* Intron.

(A) Sequences of the 30- to 36-bp DNA fragments used in EMSAs. The core TGAC motifs are boxed, while introduced mutations are in bold. Binding site 1 (BS1) is derived from region *c* of the *ga2ox1* intron (see Figure 4A) and was included for comparison because it contains a consensus in vitro binding site (Smith et al., 2002). BS2 to BS4 are derived from the region covered by primer pair *e* where KN1 binds in vivo (Figure 4).

(B) to **(G)** EMSAs were used to define the specific sequence motif bound by KN1. Unless otherwise specified, 50 ng of GST-HD proteins were used, while 250 ng of GST served as the negative control. For convenience, BS1 to BS4 DNA fragments are labeled 1 to 4, while mutated versions of BS4 are identified as C#.

(B) KN-HD binds weakly to BS1, BS2, or BS3 and binds strongly to BS4.

(C) KN-HD binds to a 570-bp DNA fragment that covers the region with the best enrichment in the ChIP assay (region *e*; see hatched box in Figure 4A). Competition assays with unlabeled double-stranded oligos (competitor) show that KN-HD binds with high affinity to a motif delimited by BS4. The presence of protein-DNA complexes of lower mobility (double circles) compared with the major complex (single circle) suggests that a fraction of the probe is bound by two molecules of KN-HD.

(D) K57V mutation (in the third helix of the HD, KN*) severely reduces the binding to BS4.

(E) to **(G)** KNOX proteins specifically bind to BS4. GN-HD and LG-HD do not bind BS4 as strongly as KN-HD, but the use of mutated DNA fragments at positions C1 and C2 as labeled probes or in competition assay show that they share the same specificity. Intensities in **(D)** and **(G)** are directly comparable since the gels were run at the same time.

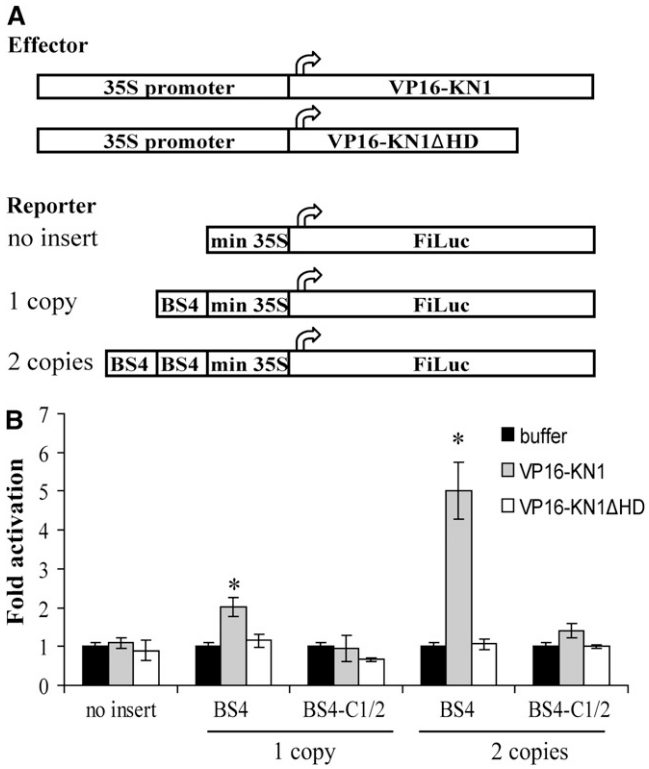

Figure 6. VP16-KN1 Modulates Luciferase Expression in Planta through a Chimeric Promoter Containing the *ga2ox1* Binding Site.

(A) and **(B)** KN1 carrying the activation domain of VP16 (VP16-KN1) or lacking the HD (VP16-KN1ΔHD) were transiently produced in vivo in *N. benthamiana* leaves along with a reporter plasmid containing a minimal 35S driving a FiLuc gene. One or two copies of BS4 or BS4-C1/2 were inserted upstream of the minimal 35S. Data were normalized with an internal RiLuc control.

(A) Cartoon representation of the constructs used for the experiment.

(B) Fold activation activity is expressed relative to the basal value obtained with the minimal 35S only for each protein and error bars represent the SD of four biological replicates (*P value < 0.01). FiLuc, Firefly luciferase; min 35S, minimal 35S promoter.

of VP16 (Triezenberg et al., 1988; Cousens et al., 1989) were cloned downstream of a full-length 35S promoter and coinfiltrated along with the reporter FiLuc plasmid and an internal control. FiLuc activity generated from the chimeric promoter was compared with that of the minimal 35S promoter in the presence of each version of KN1. When the chimeric promoter included one or two copies of BS4, VP16-KN1 induced a twofold and fivefold increase of FiLuc activity, respectively (Figure 6B). The inclusion of more than two copies of BS4 did not increase the reporter activity to more than fivefold (see Supplemental Figure 5 online). KN1 and VP16-KN1ΔHD did not activate the reporter (Figure 6B; see Supplemental Figure 5 online), nor did we observe activation with two copies of BS4-C1/2 (Figure 6B), which was not bound by KN1 in vitro (Figures 5E and 5F). These results demonstrate that the *ga2ox1* binding site is a *cis*-regulatory element sufficient for recognition and binding by KN1 in vivo. Furthermore, because no change in reporter activity could be detected until an activation domain was added to KN1, we conclude that KN1 does not act as a transcriptional activator or repressor and very likely requires a cofactor to activate or repress transcription in vivo.

The *cis*-Regulatory Element in the *ga2ox1* Intron Is Conserved across the Grasses

If KNOX regulation of *ga2ox1* is a mechanism that was recruited during evolution as a means to control bioactive GA, the KNOX binding site in the *ga2ox1* intron would likely be conserved between different species. We found putative orthologs of *ga2ox1* in rice, *Sorghum bicolor*, and *Brachypodium distachyon* based on homology and synteny with the maize genome (see Supplemental Figure 6 online). The structure of the four genes was well conserved, and multiple conserved noncoding sequences are found in the introns. The KNOX binding site is present at the end of the first intron in all three genes; however, the second TGAC motif is not perfectly conserved, with a change in either the T or the C (Figure 7A). Nucleotides at positions 1 and 4 of the TGAC motif are important for efficient KNOX binding in vitro, although they are not as critical as positions 2 and 3 (Viola and Gonzalez, 2006). However, the importance of positions 1 and 4 has never been investigated in the context of two TGAC motifs close to each other. We designed probes with mutations in one to four residues (referred to as BS4-M1 to M8, Figure 7B) and used EMSA to test the ability of KN-HD to bind. All introduced mutations reduced the binding of KN-HD to some degree, although binding to BS4-M1 and BS4-M8 was almost as strong as that of BS4 (Figure 7C). None of these mutations reduced the binding to the low level observed with BS4-C1 and BS4-C2, indicating that the most critical nucleotides for KN-HD recognition are at position 2 and 3 (GA) of the two TGAC motifs. In addition, the rice version of the binding site (BS4-*Os*, right panel in Figure 7C) produced a KN-HD binding similar to that of BS4-M6, which mimics the T-to-C substitution found in Os and Sb *ga2ox1*. This result shows that the reduced KN-HD affinity to BS4-*Os* is due mainly to the T-to-C substitution in the second TGAC motif and not to the sum of all the differences upstream and downstream of the two TGAC motifs.

Because the nucleotide substitution found in the putative KNOX binding site in Os, Sb, and Bd *ga2ox1* does not produce an in vitro KN-HD binding as strong as Zm *ga2ox1* (BS4-M5 and BS4-M6, Figure 7C), we asked whether the nucleotide substitutions found in those variants affect KN1 binding in vivo. We compared VP16-KN1's ability to activate FiLuc transcription via BS4, BS4-C1, and BS4-M6 and detected a 4.2-, 2.7-, and 5.2-fold increase, respectively, of FiLuc activity (Figure 7D). The difference between BS4 and BS4-C1 was statistically significant (P value = 0.012), but the difference between BS4 and BS4-M6 was not. These data indicate that substitutions at positions 2 and 3 of the first TGAC motif reduce KN1 affinity in vivo, but substitution at position 4 of the second TGAC motif does not. Thus, we infer that minor reduction of in vitro KNOX binding affinity does not significantly affect in vivo ability to induce the activation of a reporter gene. Therefore, variants of the *cis*-regulatory element present in Os, Sb, and Bd *ga2ox1* are likely to constitute functional KNOX binding sites in vivo.

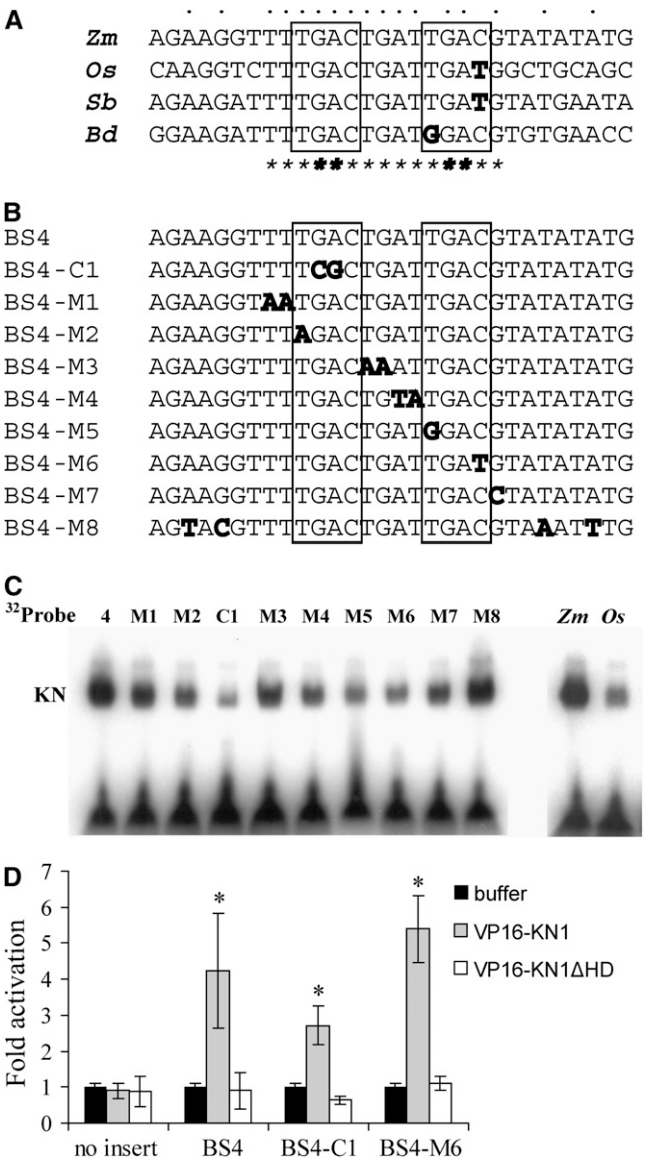

Figure 7. KNOX Binding Site in *ga2ox1* Intron Is Conserved across the Grasses.

(A) Alignment of KNOX binding site in *ga2ox1* orthologs from *Z. mays* (*Zm*), *O. sativa* (*Os*), *S. bicolor* (*Sb*), and *B. distachyon* (*Bd*), with the TGAC motifs boxed (diverging residues are in bold). Residues conserved between the four sequences are marked with a dot above the top sequence, and residues important for KNOX binding in vitro are marked with an asterisk below the bottom sequence (the most important are in bold).

(B) Sequences of the 30-bp DNA fragments used in EMSA **(C)** and in planta assay **(D)**. The core TGAC motifs are boxed, while introduced mutations are in bold.

(C) EMSA using BS4 (4) and mutated versions of BS4 (C1 and M1 to M8). Most mutations affect KN-HD binding to some degree and define a putative binding site of 15 bp. The rice version of the binding site (Os, right panel) reduces the binding affinity similarly to M5 and M6 mutations.

(D) The C1 but not M6 mutation reduces VP16-KN1 ability to activate the Firefly luciferase reporter in planta. Error bars reflect the SD of four biological replicates (*P value < 0.001).

DISCUSSION

GA is a class of plant hormones that regulate and integrate a wide range of growth and developmental processes, including germination, stem elongation, transition to flowering, pollen tube growth, and seed development (Thomas et al., 2005). Since many of these processes involve meristem function, it is likely that meristem-specific regulators, such as KNOX proteins, are involved in coordinating GA metabolism. Previous studies have suggested that GA is incompatible with meristem activities and proposed that KNOX proteins are needed to maintain low GA levels in the SAM (Hay et al., 2002; Jasinski et al., 2005). In this article, we provide evidence that KNOX-mediated downregulation of bioactive GA is mechanistically achieved at least by direct upregulation of the GA catabolism gene *ga2ox1* (Figure 8A).

We found that multiple aspects of the phenotypes conditioned by mutations at the *kn1* locus are modified when GA biosynthesis is inhibited, suggesting that KN1-mediated repression of bioactive GA is biologically relevant. One aspect involved a partial rescue of the reduced spikelet density found in the tassel of *kn1-e1*, which indicates that KN1 has a role in reducing bioactive GA during male reproductive development. This role is further supported by the occasional occurrence of pistil development in the tassel of *kn1-e1* (see Supplemental Figures 2C and 2D online). Maize flowers are initially perfect, but pistil primordia abort in the tassel and stamen primordia abort in ears (Cheng et al., 1983). GA is a feminizing hormone, and spraying experiments showed that excessive GA levels in tassels promote either male sterility or the development of pistils (Hansen et al., 1976; Rood et al., 1980). The development of pistils in staminate flowers of *kn1-e1* may represent an occasional failure to repress GA accumulation at a critical time that results in inefficient pistil abortion.

Another aspect of the *kn1* loss-of-function phenotype that involves GA is the modification of flowering time. Similar to many plant species, flowering time in maize is positively correlated with GA accumulation and signaling (Evans and Poethig, 1995; Thornsberry et al., 2001). The initiation of fewer leaves before the vegetative-to-reproductive transition in *kn1-e1* plants is possibly the result of a failure to properly downregulate GA accumulation. Our data suggest that at the molecular level, this GA downregulation occurs at least through KN1-mediated upregulation of *ga2ox1*. In rice, which also requires GA for the vegetative-to-reproductive transition, *GA2ox1* was downregulated during transition to the reproductive phase, suggesting that this downregulation could be an initial event required for development of the inflorescence meristem (Sakamoto et al., 2001b). Although we have not analyzed *ga2ox1* mRNA levels during the transition phase, the level detected in immature tassels from normal plants was four times lower than in shoot apices, and it was even lower in tassels from *kn1-e1* plants. During vegetative growth, *ga2ox1* mRNA levels did not differ between shoot apices from *kn1-e1* and normal siblings, presumably because other KNOX proteins can regulate *ga2ox1* as well. Accordingly, no defect is associated with the absence of KN1 during the vegetative phase. Thus, it is likely that in the absence of KN1, the failure to properly upregulate *ga2ox1* happens only at the end of the vegetative growth, leading to a higher GA level and a faster transition to the reproductive phase.

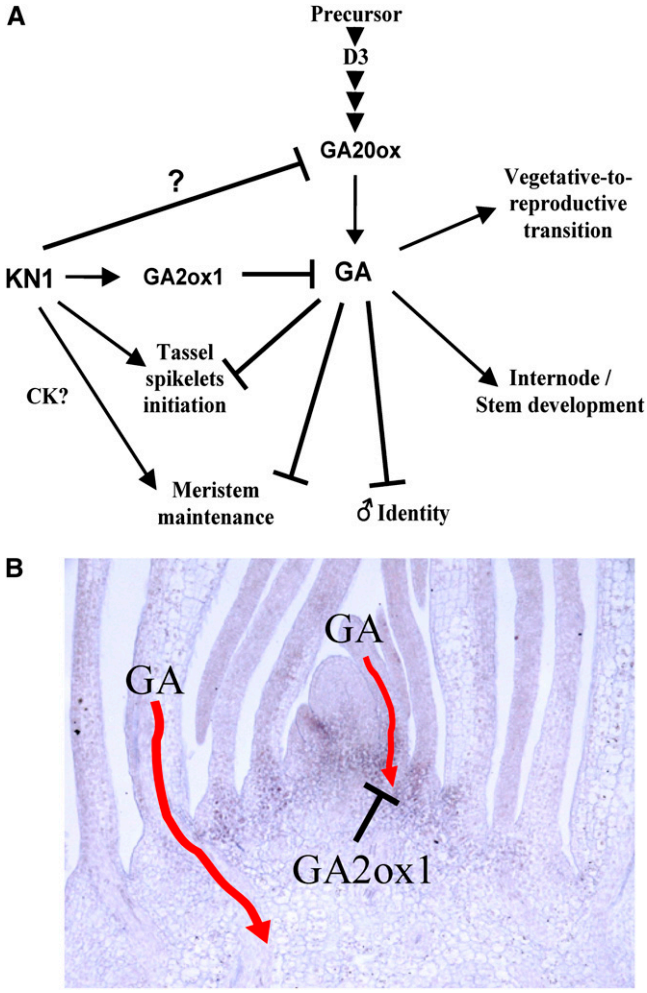

Figure 8. The Interplay between KN1 and GA Regulates Different Aspects of Maize Development.

(A) Model for the relationship between KN1, GA, and the regulation of vegetative and reproductive growth. KN1 represses the accumulation of bioactive GA directly through positive regulation of GA2ox1 and potentially by negative regulation of GA20ox (Sakamoto et al., 2001a; Chen et al., 2004), but other direct and indirect mechanisms cannot be excluded.

(B) The GA2ox1 gradient detected in the maize shoot apex presumably prevents GA synthesized in the developing leaves from entering the meristematic zone but allows GA to reach the more basal, expanding area of the stem, to favor cell expansion and differentiation.

The phenotype associated with the gain-of-function mutant *Kn1-N* was also enhanced when GA biosynthesis was inhibited, consistent with a previous report (Kessler et al., 2006). Thus, the repression of bioactive GA accumulation is instrumental to KN1 function. KN1-mediated upregulation of *ga2ox1* is one of the molecular mechanisms involved in this process. Although we cannot exclude the involvement of other mechanisms, such as repression of the GA synthesis gene encoding GA 20-oxidase, the sole increased production of GA 2-oxidase could account for the semidwarfism of *Kn1-N*. Indeed, overexpression of GA 2-oxidase genes in rice, poplar (*Populus* spp), or tobacco leads to dwarfism (Sakamoto et al., 2001b; Busov et al., 2003; Lee and Zeevaart, 2005). Conversely, loss-of-function mutations in GA 2-oxidase genes can create a hyperelongated phenotype, such as that observed in the pea (*Pisum sativum*) mutant *SLENDER* (Martin et al., 1999).

Given that *ga2ox1* is only expressed in a subset of the cells that accumulate the KN1 protein, an intriguing question is what mechanism ensures that KN1 does not promote *ga2ox1* transcription in every cell where KN1 accumulates. A likely possibility is that KN1-mediated *ga2ox1* regulation is dependent on the presence of specific cofactors that would be expressed in the same domain as *ga2ox1*. Indeed, KNOX proteins bind DNA as heterodimers with BELL proteins, another class of TALE HD proteins (Bellaoui et al., 2001; Smith et al., 2002). KNOX and BELL share similar in vitro consensus binding sites, and their heterodimerization increases their affinity for DNA (Smith et al., 2002; Viola and Gonzalez, 2006). In our in planta binding assay, no change in reporter activity could be detected until the activation domain from VP16 was added to KN1, suggesting that in vivo, KN1 modulates gene expression only in the presence of a binding partner that provides an activator or repressor module.

KNOX proteins bind DNA in a sequence-specific manner through the third helix of their HD, but their recognition sites, like their animal counterparts, are degenerate, with only a very short TGAC core consensus (Krusell et al., 1997; Smith et al., 2002; Tioni et al., 2005; Viola and Gonzalez, 2006). We found that KN1 binds to the first intron of *ga2ox1* through a *cis*-regulatory element that potentially spans ~15 bp and includes two TGAC motifs. Point mutations inside of either TGAC motif strongly reduced binding of the KNOX-HD proteins, indicating that both motifs are important for optimal binding. The presence of a tandem TGAC repeat has also been reported in potato for the binding of the BEL protein BEL5 and KNOX protein POTH1 to the promoter of the potato *ga20ox1* gene (Chen et al., 2004). Furthermore, when DNA binding motifs of KN1 or its barley homolog, HOODED, have been identified using in vitro binding assays, many of the DNA clones contained two TGAC motifs or one TGAC motif and either one TGA or GAC imperfect motif separated by 0 to 10 residues (57% of the clones in Krusell et al. [1997] and 67% in Smith et al. [2002]). In this study, we have shown that the HD of KN1 binds with a much higher affinity to naturally occurring DNA elements containing two TGAC motifs rather than only one, both in vitro and in vivo. These observations suggest that true KNOX target genes are likely to have *cis*-regulatory elements containing more than one TGAC motif.

Our data strongly suggest that KNOX transcription factors other than KN1 also modulate *ga2ox1* expression. *gn1*, *lg3*, and other maize class I *knox* genes accumulate in the SAM (Jackson et al., 1994; Schneeberger et al., 1995; Foster et al., 1999) and thus are likely to act redundantly with KN1. The upregulation of *ga2ox1* in the leaves of *Gn1-R* and *Lg3-O* and their ability to bind in vitro to the *cis*-regulatory element identified in the *ga2ox1* intron suggest that these KNOX proteins can substitute in vivo for KN1 activity. However, this substitution is certainly insufficient during the reproductive phase since development of reproductive organs is affected in *kn1* mutants. The modulation of *ga2ox1* only in tissues mostly affected by the absence of KN1 (ears and

tassels but not shoot apices) indicates that the modulation of *ga2ox1* expression by KN1 has functional significance.

Others have speculated that *ga2ox1* expression at the base of leaves prevents bioactive GA synthesized in developing leaves from diffusing to the meristematic zone where GA is thought to have a detrimental effect (Sakamoto et al., 2001b; Hay et al., 2002; Jasinski et al., 2005). Interestingly, *ga2ox1* expression in maize shoot apices follows a gradient, with strong accumulation at the base of newly initiated leaves that becomes undetectable at the base of older leaves. We propose that this differential accumulation prevents bioactive GA from reaching the meristem and the developing stem below the meristem but allows GA to reach the more basal, expanding area of the stem, to favor cell expansion and differentiation (Figure 8B).

Interestingly, *ga2ox1* orthologs from other grasses also contain a functional KNOX binding site at the end of their first intron. This finding suggests that KNOX regulation of *ga2ox1* is a mechanism that was recruited during evolution as a means to control bioactive GA levels. Furthermore, it suggests that many of the KN1/KNOX targets yet to be identified in maize very likely constitute KNOX targets in other grasses. Combined with the availability of genomes from maize and other grasses, this first identification of a true KN1/KNOX target gene in grasses opens the door to the systematic identification of KNOX targets in different grasses using KN1 as a model.

METHODS

Plant Materials and Double Mutant Analysis

Gn1-R and *kn1* alleles have been previously described (Freeling and Hake, 1985; Foster et al., 1999; Vollbrecht et al., 2000) and introgressed five times or more in the B73 background before use in this study (unless otherwise specified). *Lg3-0* (312D) and *d3-N660B* (917FH) were provided by the Maize Genetics Stock Center (accession numbers are listed after stock name). The *bd1 Tu* stock came from a cross between *bd1-2* (Chuck et al., 2002) and *Tu1-1*. Material for ChIP or Q-RT-PCR was dissected from plants grown either in our Gill Tract summer nursery (ears and tassels) or in the greenhouse (leaves, shoot apices, and tassels) in Albany, CA.

For double mutant analysis, two F2 families segregating *Kn1-N* (or *kn1-e1*) and *d3* mutations were screened at the seedling stage by visual inspection and classified as normal (*d3*/+ or +/+) or dwarf (*d3*/*d3*) based on dwarf stature. In families segregating *Kn1-N*, the *kn1* genotype was inferred from the severity of the knotted phenotype in the first leaf (*Kn1-N*/+ do not make knots and have leaves that are larger than their +/+ siblings but narrower than *Kn1-N*/*Kn1-N*). Measurements were taken 3 weeks after planting. In families segregating *kn1-e1*, all plants were genotyped by PCR for the *kn1-e1* allele and grown to maturity before tassels were measured.

ChIP

The ChIP procedure was a modified version of published protocols (Gendrel et al., 2002; Bowler et al., 2004) and is described in the Supplemental Methods online. Ears and tassels dissected for ChIP were between 0.5 and 1.5 cm. Shoot apices and leaves were dissected from 3-week-old seedlings. Shoot apices included some leaf primordia and developing stem, while leaves consisted of a segment of ~2 cm of the most inner leaves just above the SAM.

Real-Time Q-PCR

For quantification of ChIP assays, 2 μL of eluted DNA were used as template in 30-μL PCR reactions on a MyiQ single-color real-time detection system (Bio-Rad). Three technical replicates were performed for each sample, and at least two independent biological samples were analyzed for each genotype. Results were evaluated using the comparative C_T method (Livak and Schmittgen, 2001) relative to 1% of the total input, but the actual amplification efficiency of each primer pair (calculated from a standard curve run at the same time) was used instead of assuming an efficiency of 2. Primers used are specified in Supplemental Methods online.

For RT-PCR analysis, the cDNA equivalent to 75 ng of total RNA was used in a 30-μL PCR reactions on a real-time detection system described above. Data presented are the average of two to four biological replicates, each quantified with three technical replicates. Each biological replicate represents a pool of 5 to 10 individual plants, except in the case of ears (three individuals per pool). Data were normalized using Zm *gapdh* as the reference gene according to the Pfaffl method (Pfaffl, 2001). More information including primers is available in the Supplemental Methods online.

EMSA

The homeobox sequence of *KNOX* genes (representing amino acids 264 to 326 in KN1) was amplified from cloned full-length cDNA and inserted into pGEX-5X-2 to produce a translational fusion with GST protein. KN-HD* was generated by introducing a Lys-to-Val substitution at position 57 of the HD (K-320 relative to the full length protein), a mutation known to reduce the HD affinity for DNA (Viola and Gonzalez, 2006). Recombinant proteins were purified using a Glutathione Sepharose 4B resin (Amersham) and dialyzed against the storage buffer (10 mM Tris, pH 7.5, 50 mM NaCl, 0.5% Nonidet P-40, 0.5 mM EDTA, 1 mM $MgCl_2$, 10% glycerol, 0.5 mM DTT, and protease inhibitors). Radiolabeled probes were generated using [^{32}P]ATP in a T4 polynucleotide kinase reaction on double-stranded oligos or PCR products and purified with G-25 sepharose (Amersham). Normalized amount of probes (50,000 cpm for oligos and 10,000 cpm for PCR products) were incubated with 10 to 250 ng of recombinant proteins in EMSA binding buffer (10 mM Tris, pH 7.5, 50 mM NaCl, 0.15% Triton X-100, 0.5 mM EDTA, 1 mM $MgCl_2$, 4% glycerol, 0.5 mM DTT, and 100 μg/mL poly[dI.dC]) on ice for 30 min in 20-μL reaction volume. When performing a competition assay, unlabeled probes were added 30 min prior the addition of the ^{32}P-labeled probes. Binding reactions were separated on nondenaturing PAGE gels (5% polyacrylamide [60:1 ratio of acrylamide to bis-acrylamide] and 2.5% glycerol) in 0.5× Tris-borate-EDTA buffer. After electrophoresis, gels were dried and analyzed by autoradiography.

Transient in Planta Binding Assay

The firefly luciferase reporter plasmid was constructed by modifying the pTCaMV35S-F(CF) binary plasmid (Cazzonelli and Velten, 2006) according to standard DNA cloning techniques. The plasmid was cut with *Hin*dIII and *Eco*RV, which left only a minimal 35S promoter 3′ of the *Eco*RV site, blunted and used for a ligation reaction with double-stranded 30-bp oligos. The number of copies inserted was determined by sequencing. *kn1* and its variants were inserted into pBI121 downstream of the 35S promoter in place of the *gus* gene. All binary plasmids were introduced into *Agrobacterium tumefaciens* (GV3101). *A. tumefaciens* strains carrying the different plasmids were independently grown overnight, resuspended in infiltration buffer (50 mM MES, 0.5% [w/v] sucrose, 10 mM $MgCl_2$, and 0.1 mM acetosyringone, pH 5.6) to an OD_{600} of 1.0, let sit at room temperature for 2 h or more before being mixed and used for infiltration on leaves of greenhouse grown *Nicotiana benthamiana* as described (Cazzonelli and Velten, 2006). The internal control carrying the

Renilla luciferase [pE1778-SUPER-R(SR); Cazzonelli and Velten, 2006] constituted 2% of *A. tumefaciens* mixtures. Quantification of luciferase activities on infiltrated areas (~1 cm^2) was performed 2 to 3 d after infiltration using the Dual-Luciferase reporter Assay System (Promega).

Statistical Analysis

Statistical analysis of differences between samples was performed using a two-sample Student's *t* test.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: *ga2ox1* cDNA (EU973454) and genomic DNA (AC198080.3); *tub- alpha3* gene (M60171.1); and Zm *gapdh* cDNA (X07156.1). Analysis of synteny between maize and other grass genomes at the *ga2ox1* locus was performed with the *CoGe* tool (Lyons and Freeling, 2008). Genomic locations used for analysis of synteny and identification of KN1 binding site are specified in Supplemental Figure 6 online.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. *Kn1-N* Phenotype Is Enhanced by Addition of the Dominant *Dwarf9* Mutation.

Supplemental Figure 2. *kn1-e1* Phenotype Is Partially Rescued by a Low GA Level.

Supplemental Figure 3. *ga2ox1* Expression Pattern in Shoot Apices from Normal (+/+) and *kn1-e1* Plants.

Supplemental Figure 4. Immunodetection of KN1 in the Nuclear Extracts Used for ChIP.

Supplemental Figure 5. VP16-KN1 but Not KN1 Modulates Luciferase Expression in Planta.

Supplemental Figure 6. Comparison of Syntenic Regions Containing *ga2ox1* in Different Grasses.

Supplemental Table 1. Analysis of the of the *kn1-e1* Phenotype in the B73 Background.

Supplemental Methods. ChIP, RT-PCR Analysis, in Situ Hybridization, and Immunodetection and Antibodies.

ACKNOWLEDGMENTS

We thank Naomi Ori, Harley Smith, Erich Grotewold, as well as current and former members of the Hake lab for critical reading of the manuscript and helpful discussions. We are very grateful to Mario Ouellet for advice on quantitative PCR, Harley Smith who generated the KN1 serum, George Chuck who provided advice on in situ hybridization and the *bd1 Tu* genetics stock, and Jeff Velten for the gift of luciferase plasmids. We also thank Eric Lyons for his help on analysis of synteny between maize and other grasses and Annie Tang for assistance in data collection. This work was supported by National Science Foundation Grant DBI-0604923 to S.H. and grants from the U.S.-Israel Binational Science Foundation (2000109) and the U.S.-Israel Binational Agricultural Research and Development fund (IS-3453-03) to S.H. and N. Ori. N.B. was a recipient of the Fonds Québécois de la Recherche sur la Nature et les Technologies postdoctoral fellowship.

Received April 27, 2009; revised May 31, 2009; accepted June 8, 2009; published June 30, 2009.

REFERENCES

Bellaoui, M., Pidkowich, M.S., Samach, A., Kushalappa, K., Kohalmi, S.E., Modrusan, Z., Crosby, W.L., and Haughn, G.W. (2001). The *Arabidopsis* BELL1 and KNOX TALE homeodomain proteins interact through a domain conserved between plants and animals. Plant Cell **13:** 2455–2470.

Bowler, C., Benvenuto, G., Laflamme, P., Molino, D., Probst, A.V., Tariq, M., and Paszkowski, J. (2004). Chromatin techniques for plant cells. Plant J. **39:** 776–789.

Busov, V.B., Meilan, R., Pearce, D.W., Ma, C., Rood, S.B., and Strauss, S.H. (2003). Activation tagging of a dominant gibberellin catabolism gene (GA 2-oxidase) from poplar that regulates tree stature. Plant Physiol. **132:** 1283–1291.

Cazzonelli, C.I., and Velten, J. (2006). An *in vivo*, luciferase-based, *Agrobacterium*-infiltration assay system: Implications for post-transcriptional gene silencing. Planta **224:** 582–597.

Chen, H., Banerjee, A.K., and Hannapel, D.J. (2004). The tandem complex of BEL and KNOX partners is required for transcriptional repression of *ga20ox1.* Plant J. **38:** 276–284.

Cheng, P.C., Greyson, R.I., and Walden, D.B. (1983). Organ initiation and the development of unisexual flowers in the tassel and ear of *Zea mays.* Am. J. Bot. **70:** 450–462.

Chuck, G., Muszynski, M., Kellogg, E., Hake, S., and Schmidt, R.J. (2002). The control of spikelet meristem identity by the *branched silkless1* gene in maize. Science **298:** 1238–1241.

Cousens, D.J., Greaves, R., Goding, C.R., and O'Hare, P. (1989). The C-terminal 79 amino acids of the herpes simplex virus regulatory protein, Vmw65, efficiently activate transcription in yeast and mammalian cells in chimeric DNA-binding proteins. EMBO J. **8:** 2337–2342.

Evans, M.M., and Poethig, R.S. (1995). Gibberellins promote vegetative phase change and reproductive maturity in maize. Plant Physiol. **108:** 475–487.

Foster, T., Yamaguchi, J., Wong, B.C., Veit, B., and Hake, S. (1999). *Gnarley1* is a dominant mutation in the *knox4* homeobox gene affecting cell shape and identity. Plant Cell **11:** 1239–1252.

Freeling, M., and Hake, S. (1985). Developmental genetics of mutants that specify knotted leaves in maize. Genetics **111:** 617–634.

Fujioka, S., Yamane, H., Spray, C.R., Gaskin, P., Macmillan, J., Phinney, B.O., and Takahashi, N. (1988). Qualitative and quantitative analyses of gibberellins in vegetative shoots of normal, *dwarf-1*, *dwarf-2*, *dwarf-3*, and *dwarf-5* seedlings of *Zea mays* L. Plant Physiol. **88:** 1367–1372.

Gehring, W.J., Qian, Y.Q., Billeter, M., Furukubo-Tokunaga, K., Schier, A.F., Resendez-Perez, D., Affolter, M., Otting, G., and Wuthrich, K. (1994). Homeodomain-DNA recognition. Cell **78:** 211–223.

Gendrel, A.V., Lippman, Z., Yordan, C., Colot, V., and Martienssen, R.A. (2002). Dependence of heterochromatic histone H3 methylation patterns on the *Arabidopsis* gene DDM1. Science **297:** 1871–1873.

Greenboim-Wainberg, Y., Maymon, I., Borochov, R., Alvarez, J., Olszewski, N., Ori, N., Eshed, Y., and Weiss, D. (2005). Cross talk between gibberellin and cytokinin: The *Arabidopsis* GA response inhibitor SPINDLY plays a positive role in cytokinin signaling. Plant Cell **17:** 92–102.

Groover, A.T., Mansfield, S.D., DiFazio, S.P., Dupper, G., Fontana, J.R., Millar, R., and Wang, Y. (2006). The *Populus* homeobox gene *ARBORKNOX1* reveals overlapping mechanisms regulating the shoot apical meristem and the vascular cambium. Plant Mol. Biol. **61:** 917–932.

Hansen, D.J., Bellman, S.K., and Sacher, R.M. (1976). Gibberellic acid-controlled sex expression of corn tassels. Crop Sci. **16:** 371–374.

Hay, A., Kaur, H., Phillips, A., Hedden, P., Hake, S., and Tsiantis, M. (2002). The gibberellin pathway mediates KNOTTED1-type homeobox function in plants with different body plans. Curr. Biol. **12:** 1557–1565.

Jackson, D., Veit, B., and Hake, S. (1994). Expression of maize KNOTTED1 related homeobox genes in the shoot apical meristem predicts patterns of morphogenesis in the vegetative shoot. Development **120:** 405–413.

Jasinski, S., Piazza, P., Craft, J., Hay, A., Woolley, L., Rieu, I., Phillips, A., Hedden, P., and Tsiantis, M. (2005). KNOX action in *Arabidopsis* is mediated by coordinate regulation of cytokinin and gibberellin activities. Curr. Biol. **15:** 1560–1565.

Kerstetter, R.A., Laudencia-Chingcuanco, D., Smith, L.G., and Hake, S. (1997). Loss-of-function mutations in the maize homeobox gene, *knotted1*, are defective in shoot meristem maintenance. Development **124:** 3045–3054.

Kessler, S., Townsley, B., and Sinha, N. (2006). L1 division and differentiation patterns influence shoot apical meristem maintenance. Plant Physiol. **141:** 1349–1362.

Krusell, L., Rasmussen, I., and Gausing, K. (1997). DNA binding sites recognised *in vitro* by a knotted class 1 homeodomain protein encoded by the *hooded* gene, *k*, in barley (*Hordeum vulgare*). FEBS Lett. **408:** 25–29.

Lee, D.J., and Zeevaart, J.A. (2005). Molecular cloning of *GA 2-oxidase3* from spinach and its ectopic expression in *Nicotiana sylvestris.* Plant Physiol. **138:** 243–254.

Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods **25:** 402–408.

Long, J.A., Moan, E.I., Medford, J.I., and Barton, M.K. (1996). A member of the KNOTTED class of homeodomain proteins encoded by the *STM* gene of *Arabidopsis.* Nature **379:** 66–69.

Lyons, E., and Freeling, M. (2008). How to usefully compare homologous plant genes and chromosomes as DNA sequences. Plant J. **53:** 661–673.

Martin, D.N., Proebsting, W.M., and Hedden, P. (1999). The *SLENDER* gene of pea encodes a gibberellin 2-oxidase. Plant Physiol. **121:** 775–781.

Mele, G., Ori, N., Sato, Y., and Hake, S. (2003). The *knotted1*-like homeobox gene *BREVIPEDICELLUS* regulates cell differentiation by modulating metabolic pathways. Genes Dev. **17:** 2088–2093.

Muehlbauer, G.J., Fowler, J.E., Girard, L., Tyers, R., Harper, L., and Freeling, M. (1999). Ectopic expression of the maize homeobox gene *liguleless3* alters cell fates in the leaf. Plant Physiol. **119:** 651–662.

Ori, N., Juarez, M.T., Jackson, D., Yamaguchi, J., Banowetz, G.M., and Hake, S. (1999). Leaf senescence is delayed in tobacco plants expressing the maize homeobox gene *knotted1* under the control of a senescence-activated promoter. Plant Cell **11:** 1073–1080.

Pfaffl, M.W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. **29:** e45.

Phinney, B.O., and Spray, C.R. (1982). Chemical genetics and the gibberellin pathway in *Zea mays* L. In Plant Growth Substances, P.F. Wareing, ed (New York: Academic Press), pp. 101–110.

Rood, S.B., Pharis, R.P., and Major, D.J. (1980). Changes of endogenous gibberellin-like substances with sex reversal of the apical inflorescence of corn. Plant Physiol. **66:** 793–796.

Sakamoto, T., Kamiya, N., Ueguchi-Tanaka, M., Iwahori, S., and Matsuoka, M. (2001a). KNOX homeodomain protein directly suppresses the expression of a gibberellin biosynthetic gene in the tobacco shoot apical meristem. Genes Dev. **15:** 581–590.

Sakamoto, T., Kobayashi, M., Itoh, H., Tagiri, A., Kayano, T., Tanaka, H., Iwahori, S., and Matsuoka, M. (2001b). Expression of a gibberellin 2-oxidase gene around the shoot apex is related to phase transition in rice. Plant Physiol. **125:** 1508–1516.

Schneeberger, R.G., Becraft, P.W., Hake, S., and Freeling, M. (1995). Ectopic expression of the *knox* homeobox gene *rough sheath1* alters cell fate in the maize leaf. Genes Dev. **9:** 2292–2304.

Smith, H.M., Boschke, I., and Hake, S. (2002). Selective interaction of plant homeodomain proteins mediates high DNA-binding affinity. Proc. Natl. Acad. Sci. USA **99:** 9579–9584.

Smith, L.G., Greene, B., Veit, B., and Hake, S. (1992). A dominant mutation in the maize homeobox gene, *Knotted-1*, causes its ectopic expression in leaf cells with altered fates. Development **116:** 21–30.

Thomas, S.G., Rieu, I., and Steber, C.M. (2005). Gibberellin metabolism and signaling. Vitam. Horm. **72:** 289–338.

Thornsberry, J.M., Goodman, M.M., Doebley, J., Kresovich, S., Nielsen, D., and Buckler IV, E.S. (2001). *Dwarf8* polymorphisms associate with variation in flowering time. Nat. Genet. **28:** 286–289.

Tioni, M.F., Viola, I.L., Chan, R.L., and Gonzalez, D.H. (2005). Site-directed mutagenesis and footprinting analysis of the interaction of the sunflower KNOX protein HAKN1 with DNA. FEBS J. **272:** 190–202.

Triezenberg, S.J., Kingsbury, R.C., and McKnight, S.L. (1988). Functional dissection of VP16, the trans-activator of herpes simplex virus immediate early gene expression. Genes Dev. **2:** 718–729.

Viola, I.L., and Gonzalez, D.H. (2006). Interaction of the BELL-like protein ATH1 with DNA: Role of homeodomain residue 54 in specifying the different binding properties of BELL and KNOX proteins. Biol. Chem. **387:** 31–40.

Vollbrecht, E., Reiser, L., and Hake, S. (2000). Shoot meristem size is dependent on inbred background and presence of the maize homeobox gene, *knotted1.* Development **127:** 3161–3172.

Winkler, R.G., and Helentjaris, T. (1995). The maize *Dwarf3* gene encodes a cytochrome P450-mediated early step in Gibberellin biosynthesis. Plant Cell **7:** 1307–1317.

Yanai, O., Shani, E., Dolezal, K., Tarkowski, P., Sablowski, R., Sandberg, G., Samach, A., and Ori, N. (2005). *Arabidopsis* KNOXI proteins activate cytokinin biosynthesis. Curr. Biol. **15:** 1566–1571.

The Maize Transcription Factor KNOTTED1 Directly Regulates the Gibberellin Catabolism Gene ga2ox1

Nathalie Bolduc and Sarah Hake

PLANT CELL 2009;21;1647-1658; originally published online Jun 30, 2009;
DOI: 10.1105/tpc.109.068221